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Comprehensive Characterization of *Annexin I* Alterations in Esophageal Squamous Cell Carcinoma

Nan Hu,¹ Michael J. Flaig,² Hua Su,¹ Jian-Zhong Shou,³ Mark J. Roth,⁴ Wen-Jun Li,⁴ Chaoyu Wang,¹ Alisa M. Goldstein,⁵ Guang Li,⁴ Michael R. Emmert-Buck,⁶ and Philip R. Taylor¹

¹Cancer Prevention Studies Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland; ²Klinik und Poliklinik für Dermatologie und Allergologie der Ludwig-Maximilians-Universität München, Munich, Germany; ³Cancer Institute & Hospital, Chinese Academy of Medical Sciences, Beijing, People's Republic of China; ⁴Shanxi Cancer Hospital, Taiyuan, Shanxi, People's Republic of China; ⁵Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, Maryland; and ⁶Pathogenetics Unit, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland

ABSTRACT

Purpose: The purpose is to characterize alterations of the *annexin I* gene, its mRNA, and protein expression in esophageal squamous cell carcinoma.

Experimental Design: Fifty-six cases of esophageal squamous cell carcinoma were analyzed using four microsatellite markers flanking the *annexin I* gene (9q11-q21) to identify loss of heterozygosity. In addition, we performed (a) single-strand conformation polymorphism and DNA sequencing along the entire promoter sequence and coding region to identify mutations, (b) real-time quantitative reverse transcription-PCR of RNA from frozen esophageal squamous cell carcinoma tissue ($n = 37$) and *in situ* hybridization ($n = 5$) on selected cases to assess mRNA expression, and (c) immunohistochemistry ($n = 44$) to evaluate protein expression. The prevalence of the allelic variants identified in the first 56 patients was refined in 80 additional esophageal squamous cell carcinoma patients and 232 healthy individuals.

Results: Forty-six of 56 (82%) esophageal squamous cell carcinoma patients showed loss of an allele at one or more of the four microsatellite markers; however, only one (silent) mutation was seen. Two intragenic variants were identified with high frequency of allelic loss (A58G, 64%; L109L,

69%). Thirty of 37 (81%) esophageal squamous cell carcinoma patients showed reduced *annexin I* mRNA expression, which was confirmed by *in situ* hybridization, whereas *annexin I* protein expression was reduced in 79% of poorly differentiated tumor cell foci but in only 5% of well-differentiated tumor foci, although allelic loss on chromosome 9 was found in both tumor grades.

Conclusions: Allelic loss of *annexin I* occurs frequently, whereas somatic mutations are rare, suggesting that *annexin I* is not inactivated in esophageal squamous cell carcinoma via a two-hit mechanism. A decrease in *annexin I* protein expression was confirmed, consistent with a quantitative decrease in mRNA expression, and appeared to be related to tumor cell differentiation. We conclude that *annexin I* is not the tumor suppressor gene corresponding to the high levels of loss of heterozygosity observed on chromosome 9 in esophageal squamous cell carcinoma; however, dysregulation of mRNA and protein levels is associated with this tumor type.

INTRODUCTION

The *annexin I* gene (*ANXA I* or *lipocortin I*), located on chromosome 9q11-q21 (1, 2), is divided into 13 exons encoding a protein of 346 amino acid residues starting from exon 2. The binding protein encoded by *annexin I* is a pleiotropic, calcium-dependent phospholipid (3) whose ascribed functions include inhibition of phospholipase A2 (4) and mediation of apoptosis (5). It also serves as a substrate for epidermal growth factor receptor (6) and functions as a stress protein (7). Structurally, *annexin I* is a component of the cornified envelope (8) and may play an important role in keratinization. Several studies show overexpression of *annexin I* protein in epithelial malignancies, including breast cancer (9). In contrast, our studies of esophageal cancer found a high frequency of allelic loss on chromosome 9q (10, 11) and a decrease in expression of the *annexin I* protein in comparison to normal esophageal mucosa (12, 13). Thus, in esophageal squamous cell carcinoma, it seems that a loss of this gene's function may be associated with the development of esophageal squamous cell carcinoma. Thus far, the majority of *annexin I* studies have focused on protein expression; mutation analysis, including germ-line and somatic, have not been reported in tumors, although one study tested for germ-line mutations in 41 patients with type 2 diabetes (14). The objective of the current study was to elucidate potential mechanisms responsible for the reported decrease in *annexin I* expression in esophageal squamous cell carcinoma by analyzing allelic loss, DNA mutations, and alterations in mRNA and protein expression in these tumors.

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Requests for reprints: Philip R. Taylor, Cancer Prevention Studies Branch, National Cancer Institute, 6116 Executive Plaza, Room 705, Bethesda, MD 20892-8314. Phone: (301) 594-2932; Fax: (301) 435-8645; E-mail: ptaylor@mail.nih.gov.

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MATERIALS AND METHODS

Overall Patient Selection. Patients who presented between 1995 and 1999 to the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, People's Republic of China, and were diagnosed with esophageal squamous cell carcinoma and considered candidates for curative surgical resection, were identified and recruited to participate in this study. All patients had a histologic diagnosis of esophageal squamous cell carcinoma confirmed by pathologists at both the Shanxi Cancer Hospital and the National Cancer Institute; no patients had prior therapy, and Shanxi was the ancestral home for all patients. The Institutional Review Boards of the Shanxi Cancer Hospital and the United States National Cancer Institute approved the study.

Patients for Genomic Testing. Fifty-six esophageal squamous cell carcinoma patients were selected to test for loss of heterozygosity (LOH) at loci flanking *annexin I* using four microsatellite markers. In addition, single-strand conformation polymorphism (SSCP) plus DNA sequencing were performed to identify variants along the entire coding sequence. The demographic characteristics of these original 56 esophageal squamous cell carcinoma patients were previously described (15) along with *TP53*, *BRCA2*, and *DICE1* gene mutation results (16–18). Eighty additional esophageal squamous cell carcinoma patients and 232 healthy individuals (101 from Beijing and 131 from Yangcheng in Shanxi Province) were specifically analyzed for germ-line variants identified in the initial 56 esophageal squamous cell carcinomas. Details of this set of patients and healthy individuals were also reported previously (18).

Patients for Expression Analysis. RNA expression using real-time reverse transcription-PCR (RT-PCR) was performed on a separate set of 37 patients with archived frozen tumor.

Patients for Protein Analysis. An annexin I IHC antibody was used to evaluate protein expression in 44 of the original 56 cases in which there was sufficient paraffin block material available.

Biological Specimen Collection and Processing. Ten milliliter of venous blood was taken from each patient before surgery, and genomic DNA was extracted and purified using standard methods. Tumor tissue obtained during surgery was fixed in ethanol or formalin and embedded in paraffin or snap-frozen in liquid nitrogen and stored at -80°C until used.

Laser Capture Microdissection (LCM) and Extraction of Tumor DNA. Tumor cells were harvested using LCM and DNA was extracted using methods described previously (15, 19).

Microsatellite Marker, PCR, and LOH Analysis. Four microsatellite markers, D9S166 (233–261 bp), D9S1822 (157–163 bp), D9S1876 (132–156 bp), and D9S175 (200–261 bp; Human MapPairs; Research Genetics, Huntsville, AL) were used to evaluate the regions flanking the *annexin I* gene (located between D9S1822 and D9S1876 for LOH.⁷ The distances separating the four microsatellite markers evaluated are as follows: 0.5 cM between D9S166 and D9S1822; 1.0 cM between D9S1822 and D9S1976; and 2.0 cM between D9S1876 and

D9S175. PCR reactions, LOH gel methods, and interpretations were performed as described previously (15).

Mutational Testing By SSCP and DNA Sequencing. PCR primers for the promoter region of *annexin I* gene and all 13 of its exons, including intron/exon boundaries, were designed according to the GenBank nucleotide sequences (accession numbers 12735231 and 27482052 for exon 2 to exon 13 and U25414 for promoter region and exon 1). Six pairs of primers were used to cover the promoter region, excluding the first 49 nucleotides that form several hairpin loops and dimers that are less amenable to PCR amplification (Table 1). Microdissected tumor DNA was obtained from resection specimens, and genomic DNA was extracted from venous blood. PCR reactions were carried out using a 10- μL final volume containing 1.0 μL of $10\times$ PCR buffer I [100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, and 15 mmol/L MgCl_2], 1.0 μL of 2.5 mmol/L deoxynucleoside triphosphate, 2 μL of DNA extraction buffer, 0.2 μL of each primer, 0.10 μL of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA), and 1 μCi of [α - ^{32}P] dCTP. Typical PCR conditions were as follows: 10 minutes of denaturation at 94°C , followed by 35 1-minute cycles at 94°C , then 51°C to 58°C for 1 minute, and finally 72°C for 1 minute. An elongation step at 72°C for 10 minutes was added to the final cycle for each exon and the promoter region. Labeled PCR products were run on SSCP gels, and the size of each PCR product is shown in Table 1. SSCP and DNA sequencing analysis were conducted according to previously described methods (16).

Total RNA Extraction, Quantitative Real-Time RT-PCR, and Relative Standard Curve. Total RNA was extracted from frozen tumor and matched normal tissues using a TRIzol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's protocol. The quality and quantity of RNA were determined with an RNA 6000 Labchip/Aligent 2100 Bioanalyzer (Agilent Technologies, Germantown, MD) or by electrophoresis with a 1.2% denaturing agarose gel and spectrophotometer. Extracted RNA was purified using RNeasy Mini kit (Qiagen, Inc., Valencia, CA) and Rnase-Free Dnase Set digestion (Qiagen, Inc.). Reverse transcription of RNA was performed by adding 5 μg of total RNA, 1 μL of oligo(dT)_{12–18} (500 $\mu\text{g}/\text{mL}$), 1 μL (200 units) of the Superscript II reverse transcriptase, 1 μL (2 units) of *Escherichia coli* Rnase, and 1 μL 10 mmol/L deoxynucleoside triphosphate (Invitrogen, Carlsbad, CA). All real-time PCR reactions were performed using an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). Primers and probes for the target (*annexin I*) and internal control genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed by Perkin-Elmer Applied Biosystems. A singleplex reaction mix was prepared according to the manufacture's protocol of "Assays-on-Demand Gene Expression Products" and included 10 μL of TaqMan Universal PCR Master mix, No AmpErase UNG ($2\times$), 1 μL of $20\times$ Assays-on-Demand Gene Expression Assay Mix (all gene expression assays have a carboxyfluorescein reporter dye at the 5'-end of the TaqMan minor groove binder probe and a non-fluorescent quencher at the 3'-end of the probe), and 9 μL of cDNA (90 ng) diluted in Rnase-free water, in a total 20 μL volume. Every sample was run in triplicate. Thermal cycling conditions consisted of 10 minutes at 95°C denaturation step, 40

⁷ Internet address: <http://www.ncbi.nlm.nih.gov/genemap99>, G3map.

Table 1 Primer sequences for annexin I

Exon	Primer name	Sequences	Annealing temperature (°C)	Run time (hours) 6 W	Size of PCR at product (size of exon) (bp)
1	anex-E1a	5'-gttgctaggtgtggcttcc-3'	56	20	203 (60)
	anex-E1b	5'-tgtagaagcagtaacattt-3'			
2	anex-E2a	5'-ggtaggaaggagaggttgt-3'	51	20	240 (80)
	anex-E2b	5'-ctaagaatgagagagaaata-3'			
3	anex-E3a	5'-gtaagtagagtataataa-3'	51	22	265 (109)
	anex-E3b	5'-acagtcattcaaatagccat-3'			
4	anex-E4a	5'-tgtcattctcccaagtgc-3'	58	22	350 (95)
	anex-E4b	5'-taactgtgcttccgaatcca-3'			
5	anex-E5a	5'-tagcatgttactattggaa-3'	58	20	220 (114)
	anex-E5b	5'-gtg acttgcccaatacatcc-3'			
6	anex-E6a	5'-aaactcattgatcctctgc-3'	55	20	243 (91)
	anex-E6b	5'-attaccatagacagtcaat-3'			
7	anex-E7a	5'-ttcaaccaacttagagatgt-3'	58	20	236 (80)
	anex-E7b	5'-atagcaacacaaaatggact-3'			
8	anex-E8a	5'-agtaaaatctgtatctgag-3'	56	24	256 (57)
	anex-E8b	5'-ttaaggtaggaagatgaactg-3'			
9	anex-E9a	5'-cttattgtgactactctga-3'	55	22	264 (94)
	anex-E9b	5'-ttccataaacagattccaga-3'			
10	anex-E10a	5'-gcattgtatcttagttgaat-3'	55	20	256 (96)
	anex-E10b	5'-gatgcactactctgattgtt-3'			
11	anex-E11a	5'-gtgaagaatgatgatgagg-3'	58	20	249 (59)
	anex-E11b	5'-gagggtttctgttctttctac-3'			
12	anex-E12a	5'-tgaatatgagacacttacc-3'	55	20	249 (123)
	anex-E12b	5'-tagatacaataagtaactc-3'			
13	anex-E13a	5'-gtgaatggtaatgttaatctc-3'	51	20	244 (57)
	anex-E13b	5'-agcttataggatgaaat-3'			
Promoter					
1	anex-p1a	5'-ttctatgtaactgacctattt-3'	52	19	188
	anex-p1b	5'-acgactttgttcctaga-3'			
2	anex-p2a	5'-tttggcattacctttgtca-3'	52	20	224
	anex-p2b	5'-tatcaagttgtctaccac-3'			
3	anex-p3a	5'-tgttattttctcattgtcac-3'	52	20	240
	anex-p3b	5'-tgttgtctttttccccgcct-3'			
4	anex-p4a	5'-ggagtttgagacctgect-3'	56		259
	anex-p4b	5'-cccaatcctaataaccagtg-3'			
5	anex-p5a	5'-agtctacaacctatttaac-3'	52	20	237
	anex-p5b	5'-ggctcagctatgtccaaa-3'			
6	anex-p6a	5'-cctttgtaatgccagttgaa-3'	52	20	240
	anex-p6b	5'-cctttatctctaccttctg-3'			

15-second cycles at 95°C, and 1 minute at 60°C. Each sample was serially (100, 10, 1, 0.1, and 0.01 ng) diluted 10-fold for real-time PCR analysis in accordance with the standard protocol provided by Perkin-Elmer Applied Biosystems. A relative standard curve quantitation method was performed as described previously (20, 21).⁸ The PCR efficiency (E) was calculated using the formula, $E = 10^{(1/\text{slope})} - 1$, and ranged from 90 to 100% for our assays (21). For an example, see Fig. 1.

Comparative C_T Method (2^{- $\Delta\Delta C_T$) for Relative Quantitation of Gene Expression.} The mean *annexin I* mRNA expression level for the three real-time quantitative RT-PCR experiments was calculated for each case. Results of the real-time RT-PCR data are presented as C_T values, where C_T is defined as the threshold PCR cycle number at which an amplified product is first detected. There is an inverse correlation between C_T and amount of target: lower target amounts correspond to higher C_T values and visa versa. The average C_T was calculated for both

annexin I and *GAPDH* and the ΔC_T was determined as the mean of the triplicate C_T values for *annexin I* minus the mean of the triplicate C_T values for *GAPDH*. The $\Delta\Delta C_T$ represents the difference between the paired tissue samples, as calculated by the formula $\Delta\Delta C_T = (\Delta C_T \text{ of tumor} - \Delta C_T \text{ of normal})$. The N-fold differential expression of the *annexin I* gene for a tumor sample compared with its normal epithelial counterpart was expressed as $2^{-\Delta\Delta C_T}$ (20, 22).⁷ Using the $2^{-\Delta\Delta C_T}$ method, the data are presented as the fold change in the target gene (*annexin I*) expression in tumor normalized to an internal control gene (*GAPDH*) and relative to the nontumor control.

In situ Hybridization. *In situ* hybridization was carried out according to standard protocols by Molecular Histology Labs, Inc. (Montgomery Village, MD). Radioactive sense and antisense probes were synthesized using IMAGE clone 2459615, MGC ID 5095.⁹ Hybridization was carried out on

⁸ Internet address: <http://docs.appliedbiosystems.com/search>.

⁹ Internet address: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=nucleotide&list_uids=12654862&dopt=GenBank.

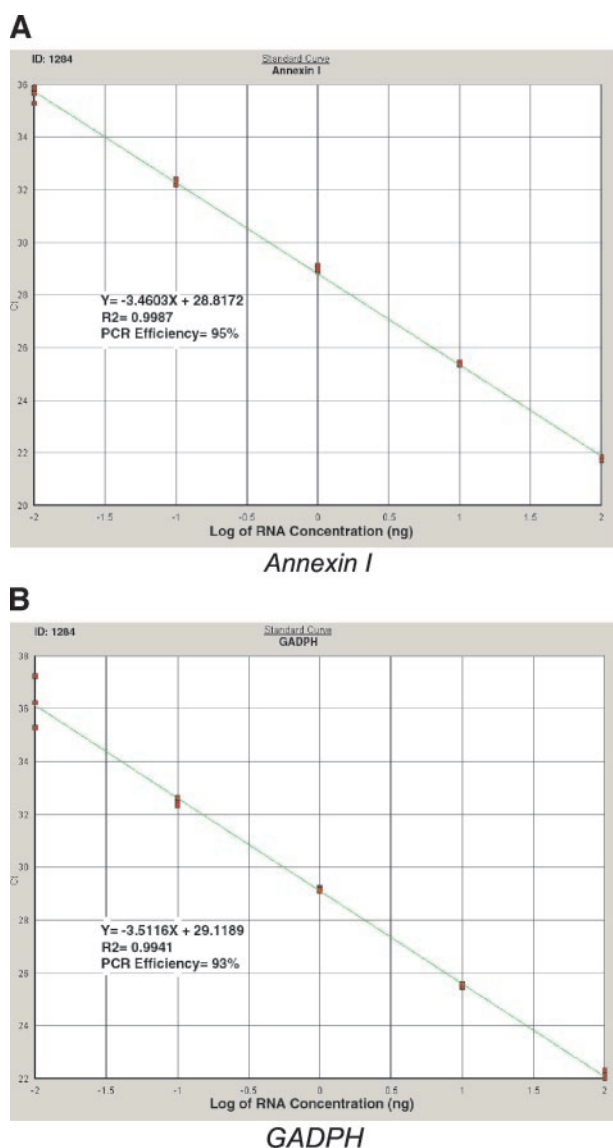


Fig. 1 The relative standard curve using the *annexin I* gene (A) and *GAPDH* (B) primers and probes amplified with 100, 10, 1, 0.1, and 0.01 ng of total RNA. Each sample was run in triplicate (red square). The average C_T values (y axis) are plotted against the logarithm of the input amount of RNA (x axis) added to each sample. PCR efficiency = $(10^{-1/\text{slope}} - 1)$. Both A and B show a linear relationship between RNA concentration and the C_T value of reverse transcription real-time PCR reaction for *annexin I* and *GAPDH*.

formalin-fixed, paraffin-embedded tissue sections after dewaxing, rehydration, blocking, and prehybridization. Parallel sections were hybridized with sense and antisense probes. Visualization was achieved with emulsion autoradiography and staining with H&E.

IHC Analysis. IHC was performed using annexin I antibodies according to the manufacturer's protocols (Transduction Laboratories, Lexington, KY). In brief, 5- μ m thick deparaffinized tissue sections were pretreated with 3% H_2O_2 in methanol for 10 minutes followed by 1 hour in 10% normal goat

serum to block endogenous peroxidase activity. Each section was incubated overnight at 4°C with a primary anti-annexin I monoclonal antibody (1:200 dilution; Transduction Laboratories), then exposed at room temperature to a biotinylated anti-mouse monoclonal antibody (1:500 dilution) and, finally, an ABC solution (Vector Laboratories, Burlingame, CA), each for 1 hour. Slides were developed with 0.02% 3',3'-diaminobenzidine solution (Sigma), counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene. The presence of well-differentiated, poorly differentiated, and carcinoma *in situ* (CIS) foci in each tumor was determined along with the staining intensity of each component. Well-differentiated foci generally consisted of cells with low nuclear-to-cytoplasmic ratios, approximating that seen in histologically normal appearing cells, and hard or dense appearing cytoplasm consistent with squamous differentiation. Squamous pearls, or mature appearing cells forming concentric rings, were focally identified in association with well-differentiated areas. Poorly differentiated regions were generally composed of cells with high nuclear-to-cytoplasmic ratios and less mature appearing cytoplasm. CIS foci consisted of dysplastic epithelial changes involving the entire thickness of the squamous mucosa without extension through the basement membrane.

Immunohistochemical-LCM LOH Analysis. Well-differentiated and poorly differentiated cellular foci were selected from *annexin I* immunohistochemically stained slides and microdissected by LCM (400 to 1100 shots, 15 to 30- μ m pulse diameters). Unstained, ethanol-fixed, paraffin-embedded 5- μ m thick histologic tissue sections were prepared on glass slides, deparaffinized three times with xylene, rinsed with 100, 95, and 70% ethanol, immunostained with anti-annexin I monoclonal antibody (1:100 dilution; Transduction Laboratories) using the EnVision polymer technique on a Dako Autostainer. Counterstaining was performed with Mayer's hematoxylin (Biogenex), after rinses with 70, 95, 100% ethanol, xylene, and air drying. The cells obtained were resuspended in 30 to 90 μ L solution containing 0.01 mol/L Tris-HCl, 1 mmol/L EDTA, 1% Tween 20, and 0.1 mg/mL proteinase K (pH 8.0) and incubated overnight at 55°C. The mixture was then boiled for 8 minutes to inactivate the proteinase K at 95°C. Two microliters of this solution were used for each PCR reaction. PCR reactions and data analysis were carried out as described above.

RESULTS

LOH Analysis of Chromosomal Regions Adjacent to *Annexin I*

Forty-six of 56 (82%) esophageal squamous cell carcinoma patients lost an allele at one or more of the four microsatellite markers flanking the *annexin I* gene; 70% of these cases (39 of 56) had LOH at all of their informative loci. Eight patients showed no LOH and 2 patients were homozygous (uninformative) at all four markers. The LOH frequencies were 88% (21 of 24), 83% (30 of 36), 78% (28 of 36), and 76% (29 of 38) for D9S166, D9S1822, D9S1876, and D9S175 in informative cases, respectively (Table 2).

Table 2 Summary of LOH on chromosome 9q, alterations in the *annexin I* gene, and annexin I protein expression in esophageal squamous cell carcinoma patients

No.	Patient ID	Lymph node metastasis (Y/N)	Allelic loss*				Frequency of LOH	Alteration in <i>annexin I</i> †		Annexin I protein expression using IHC
			D9S166	D9S1822	D9S1876	D9S175		Exon 1	Exon 5	
1	SHE069	N	H	L	H	L	2/2	1 > Y/LP	1 > Y/R	+/-
2	SHE080	N	L	L	L	H	3/3	Y/LW	Y/LW	-/focal+
3	SHE095	N	H	R	R	H	0/2	Y/R	Y/R	+/-
4	SHE096	N	H	L	R	R	1/3	Y/R	Y/LW	NA
5	SHE123	NA	H	H	H	L	1/1	Y/LW	Y/LW	+/-
6	SHE152	N	H	H	R	H	0/1	Y/R	Y/LP	+/-NP
7	SHE170	N	H	H	R	R	0/2	Y/R	Y/R	NP/strong +
8	SHE186	N	L	L	L	L	4/4	Y/LP	Y/LP	+/-
9	SHE198	Y	L	L	H	H	2/2	Y/LW	Y/LW	+/-
10	SHE208	N	H	H	H	R	0/1	Y/<22 6>	Y/LP	NP/rare +
11	SHE297	Y	H	L	L	L	3/3	N	Y/LW	+/+
12	SHE328	N	H	H	L	L	2/2	Y/LP	Y/R	+/-, CIS -
13	SHE340	Y	L	H	L	L	3/3	Y/LP	Y/LP	+/-NP
14	SHE384	N	H	L	H	L	2/2	Y/LW	Y/LW	+/-, CIS +
15	SHE459	N	R	R	H	H	0/2	Y/R	Y/R	+/-
16	SHE480	Y	H	L	L	L	3/3	Y/LW	Y/LW	NA
17	SHE240	Y	L	L	L	H	3/3	HP	HP	NA
18	SHE021	N	L	R	R	H	1/3	N	N	NA
19	SHE027	N	L	L	R	L	3/4	N	N	+/-, CIS +
20	SHE034	Y	L	H	L	L	3/3	N	N	+/-weak+
21	SHE052	N	H	L	L	L	3/3	N	N	+/-, CIS+
22	SHE057	Y	H	L	L	L	3/3	N	N	+/-
23	SHE066	Y	H	H	L	L	2/2	N	N	+/-
24	SHE081	N	H	L	H	R	1/2	N	N	+/-
25	SHE083	N	L	H	L	L	3/3	N	N	+/-
26	SHE093	N	H	H	L	L	2/2	N	N	NA
27	SHE098	N	H	H	L	L	2/2	N	N	+/+
28	SHE108	Y	H	H	H	L	1/1	N	N	NP/weak+, DYS+
29	SHE109	Y	L	H	H	L	2/2	N	N	NA
30	SHE113	N	H	L	L	L	3/3	N	N	+/+, DYS+
31	SHE118	Y	H	L	H	H	1/1	N	N	NA
32	SHE138	N	R	R	H	R	0/3	N	N	NP/weak+, CIS weak+
33	SHE150	N	H	H	L	L	2/2	N	N	NA
34	SHE200	Y	0	L	L	L	3/3	N	N	+/+
35	SHE216	N	L	H	L	H	2/2	N	N	NP/+
36	SHE235	N	H	R	R	R	0/3	N	N	+/-
37	SHE247	Y	L	L	L	L	4/4	N	N	+/-
38	SHE252	Y	L	L	L	L	4/4	N	N	+/-
39	SHE261	N	L	L	L	L	4/4	N	N	NA
40	SHE263	NA	L	L	H	L	3/3	N	N	NA
41	SHE265	N	L	L	H	H	2/2	N	N	NP/+
42	SHE273	Y	H	H	H	L	1/1	N	N	rare+/-NP
43	SHE308	Y	H	R	R	R	0/3	N	N	+/-
44	SHE322	N	L	L	H	H	2/2	N	N	+/-, CIS+
45	SHE360	Y	H	L	L	H	2/2	N	N	+/-
46	SHE391	N	L	L	H	H	2/2	N	N	rare+/-
47	SHE408	N	H	L	H	R	1/2	N	N	NA
48	SHE409	N	H	L	L	R	2/3	N	N	+/-
49	SHE437	N	H	L	L	H	2/2	N	N	rare+/-
50	SHE444	N	H	L	L	H	2/2	N	N	-/-
51	SHE488	N	H	H	H	H	0/0	N	N	+/-, CIS -
52	SHE495	Y	L	L	L	L	4/4	N	N	+/-
53	SHE497	Y	H	H	L	L	2/2	N	N	+/-
54	SHE507	Y	L	H	H	L	2/2	N	N	+/-, CIS -
55	SHE510	Y	R	L	H	L	2/3	N	N	+/-
56	SHE516	N	H	H	H	H	0/0	N	N	NA

NOTE. When informative, areas of CIS and dysplasia (DYS) are also evaluated. Qualitative remarks, such as rare or weak, are included where necessary.

* Allelic loss: R, retention; H, homozygous; L, loss of heterozygosity.

† Alteration in *annexin I*: Y, with genetic alteration; N, without genetic alteration; LP, lost polymorphic (variant) allele; LW, lost wild-type allele; NA, data not available; R, retention; HP, homozygous for polymorphism.

‡ Immunohistochemistry: WD, well-differentiated; PD, poorly differentiated; NA, data not available; positive (+), versus negative (-) staining is presented for each category; NP, tissue type not present.

Mutation and Intragenic Allelic Loss in the *Annexin I* Gene

SSCP analysis and DNA sequencing of the entire coding sequence for *annexin I*, including 13 exons, exon/intron boundaries, and all but 49 bp of its promoter region, identified band shifts in exon 1 (16 of 56, 29%) and exon 5 (17 of 56, 30%) and a single case with an anomalous band in exon 3. No mutations or polymorphisms were found in the promoter region. Somatic mutation of *annexin I* was rare in the esophageal squamous cell carcinoma patients despite the high frequencies of LOH for the four flanking polymorphic markers. One case (SHE340) did have a silent germ-line mutation (GCG→GCA resulting in Ala→Ala) at codon 35 of exon 3 and a somatic loss of the wild-type allele at the same loci. This case also contained intragenic allelic losses at exons 1 and 5 and LOH at all three informative microsatellite markers.

Genomic DNA sequencing of abnormal SSCP bands identified in 16 cases, including 15 heterozygotes and a single case homozygous for the variant allele (SHE240), showed a single A→G nucleotide change at NT5 in exon 1 (A78G), consistent with the reported polymorphic variation at this site (NCBI SNP Cluster ID: rs1131072). Seventy-one percent of the cases (40 of 56) were homozygous for the wild-type genotype (AA), 27% (15 of 56) were heterozygous (AG), and one (2%) was homozygous for the variant (GG), resulting in allele frequencies of 0.85 and 0.15 for A and G, respectively. Nine of 14 heterozygous cases (64%; one failed PCR amplification) had allelic loss, including 5 cases that lost a wild-type allele and 4 cases that lost a variant allele (Table 2).

DNA sequence analysis of exon 5 identified a single polymorphic A→G nucleotide substitution without an amino acid change (L109L). Seventy percent (39 of 56) of the cases were homozygous for the wild-type (TTA/TTA), 29% (16 of 56) were heterozygous (TTA/TTG), and 1 case (SHE240; 2%) was homozygous for the variant (TTG/TTG), resulting in allele frequencies of 0.84 and 0.16 for the TTA and TTG alleles, respectively. Eleven of 16 informative cases (69%) showed allelic loss in their tumor, including 7 who lost a wild-type allele and 4 who lost a variant allele (Table 2). Fifteen of 16 (94%) cases were informative at both A58G and L109L, and 7 of these cases had intragenic allelic loss at both sites (Table 2).

To additionally refine the allelic frequencies for the polymorphic loci at exon 1 A58G and exon 5 L109L in this high-risk Chinese population, we analyzed an additional 80 esophageal squamous cell carcinoma patients and 232 healthy individuals. The genotype frequencies for the A58G polymorphism for all esophageal squamous cell carcinoma patients tested ($n = 136$) were 61% homozygous wild-type (AA), 35% heterozygous (AG), and 4% homozygous variants (GG); allele frequencies were 0.79 and 0.21 for the wild-type (A) and variant (G) alleles, respectively. The corresponding genotypic frequencies for the healthy individuals were similar: 65% AA, 31% AG, and 4% GG, with allele frequencies of 0.81 and 0.19 for the A and G alleles, respectively (esophageal squamous cell carcinoma *versus* healthy individual allele frequencies; Fisher's exact test, $P = 0.71$). Analysis of the L109L genetic polymorphism showed that esophageal squamous cell carcinoma patients and healthy individuals had identical genotype frequencies [63%

homozygous wild-type, 33% heterozygous, 4% homozygous variants; allele frequencies of wild-type (AA), 80%, and variant (GG), 20%].

Table 2 shows the cases with intragenic allelic loss and LOH on four microsatellite markers. Only LOH at D9S1876 was significantly associated with allelic loss at A58G in exon 1 ($P = 0.03$, two-sided Fisher's exact test), but the case numbers are small (four had allelic loss at both A58G and D9S1876 *versus* four with loss at neither).

mRNA Expression of *Annexin I*

Quantitative Real-Time RT-PCR. There was a strong linear relationship between the C_T and the log of the RNA concentration of the template ($R^2 > 0.99$; Fig. 1). PCR efficiency ranged from 92 to 95% for the assays. Eighty-one percent (30 of 37) of the tumors showed a decrease in mRNA expression (median = 0.118, range 0.0006 to 0.6877 as ratio of expression in tumor compared with normal epithelium). In comparison to histologic normal, 19% showed increased expression (median ratio = 13.99, range 1.5121 to 172.4459; Table 3 and Fig. 2). The means between samples characterized as well and poorly differentiated did not differ because there were admixtures of both cell types in each sample.

***In situ* Hybridization.** *Annexin I* mRNA levels as assessed by *in situ* hybridization showed that RNA expression paralleled the cytologic maturation of the squamous epithelium from the basally located less differentiated cells to the more differentiated mucosally located cells (Figs. 3 and 4). Consequently, poorly differentiated tumor cell foci were negative for *annexin I* mRNA expression, and the well-differentiated foci were positive. In general, *annexin I* mRNA expression correlated closely with protein expression as assessed by IHC.

Annexin I Protein Expression

Immunohistochemistry. In general, annexin I protein expression was significantly reduced in the majority of esophageal squamous cell carcinoma. However, protein expression tended to vary with tumor differentiation. Thirty-eight of 44 (86%) esophageal squamous cell carcinoma cases had foci of well-differentiated tumor cells, and nearly all of these (36 of 38 or 95%) showed positive staining for annexin I protein. Forty-one of 44 (93%) contained foci of poorly differentiated tumor cells, and 29% (12 of 41) of these foci showed positive staining for this protein (Fig. 3). Most of the cases (35 of 44, 80%) contained both well-differentiated and poorly differentiated tumor cells with positive staining largely limited to the well-differentiated component (Table 2). Foci of CIS were present in eight of these cases, and five (62%) reacted positively with antibodies to the annexin I protein. Two dysplastic foci also stained positive.

Immunohistochemical and LCM LOH Analysis. To investigate the relationship between genomic alterations and annexin I protein expression, we microdissected both IHC-positive and -negative regions of five tumors and evaluated LOH at two microsatellite markers on chromosome 9 located centromeric and telomeric to the gene (Table 4). In all cases, both regions of each tumor showed allelic loss in at least one marker.

Table 3 RNA expression of *annexin 1* in 37 esophageal squamous cell carcinoma cases

A. Summary of RNA expression changes										
ID SHE	Pathology grade*	N-fold changes 2- $\Delta\Delta\text{Ct}$								
1190	PD	0.0006	B. Case 1190 detailed							
1433	PD	0.0025	1190	<i>GAPDH</i> (G)	Mean (G)	<i>Annexin 1</i> (A)	Mean (A)	ΔCt (A–G)	$\Delta\Delta\text{Ct}$ (T–N)	2- $\Delta\Delta\text{Ct}$
1450	PD	0.0031	1190N	22.26		20.91				
891	PD	0.0131	1190N	22.25		21.05				
1447	PD	0.0136	1190N	22.19	22.23	21.07	21.01	–1.22		
1423	PD	0.0151	1190T	19.07		28.49				
1279	PD	0.0256	1190T	19.08		28.54				
1258	WD	0.0262	1190T	19.03	19.06	28.38	28.47	9.41	10.63	0.0006
1250	PD	0.0278								
1184	PD	0.0301								
1277	PD	0.0562								
1464	WD	0.0606								
1157	WD	0.0852								
1446	PD	0.0937								
1169	PD	0.1090								
1401	WD	0.1268								
1418	PD	0.1283	C. Case 1400 detailed							
1241	WD	0.1337	1400	<i>GAPDH</i> (G)	Mean (G)	<i>Annexin 1</i> (A)	Mean (A)	ΔCt (A–G)	$\Delta\Delta\text{Ct}$ (T–N)	2- $\Delta\Delta\text{Ct}$
1479	PD	0.1353	1400N	20.56		19.40				
1201	WD	0.1481	1400N	20.56		19.64				
1163	WD	0.1530	1400N	20.52	20.54	19.55	19.53	–1.01		
1489	PD	0.1550	1400T	21.70		21.25				
1199	PD	0.2606	1400T	21.76		21.22				
1415	WD	0.3015	1400T	21.68	21.71	21.27	21.25	–0.47	0.54	0.69
1256	WD	0.3882								
1478	PD	0.4205								
1242	PD	0.4644								
1451	PD	0.6189								
1203	WD	0.6708								
1400	PD	0.6877								
1488	WD	1.5121	D. Case 1192 detailed							
1448	WD	2.3545	1192	<i>GAPDH</i> (G)	Mean (G)	<i>Annexin 1</i> (A)	Mean (A)	ΔCt (A–G)	$\Delta\Delta\text{Ct}$ (T–N)	2- $\Delta\Delta\text{Ct}$
1188	WD	10.1972	1192N	23.80		29.36				
1432	PD	13.9936	1192N	23.76		29.43				
1475	PD	15.7066	1192N	23.69	23.75	29.35	29.38	5.63		
1289	PD	20.6776	1192T	21.80		19.94				
1192	PD	172.4459	1192T	21.82		20.02				
			1192T	21.81	21.81	20.07	20.01	–1.80	–7.43	172.45

* Pathology grade: WD, well-differentiated; PD, poorly differentiated.

DISCUSSION

In previous reports, we described a decrease in annexin I protein expression in esophageal squamous cell carcinoma in comparison to matched normal esophageal mucosa (12, 13). To elucidate potential mechanisms for this decrease, the current study examined the genomic, transcriptional, and protein alterations of *annexin 1* in esophageal squamous cell carcinoma tumors. LOH of chromosomal regions flanking *annexin 1* was frequent as was intragenic allelic loss at two polymorphic sites.

In addition, we identified an association between allelic loss at the A58G polymorphism and D9S1876, a neighboring microsatellite marker, plus a single silent mutation. However, no significant relationship between genomic alterations on chromosome 9 and *annexin 1* expression was determined.

The results presented here represent the first report of genotype and allele frequencies for two SNPs in *annexin 1* (G58A and L109L) in esophageal squamous cell carcinoma patients and healthy individuals from a high-risk population in

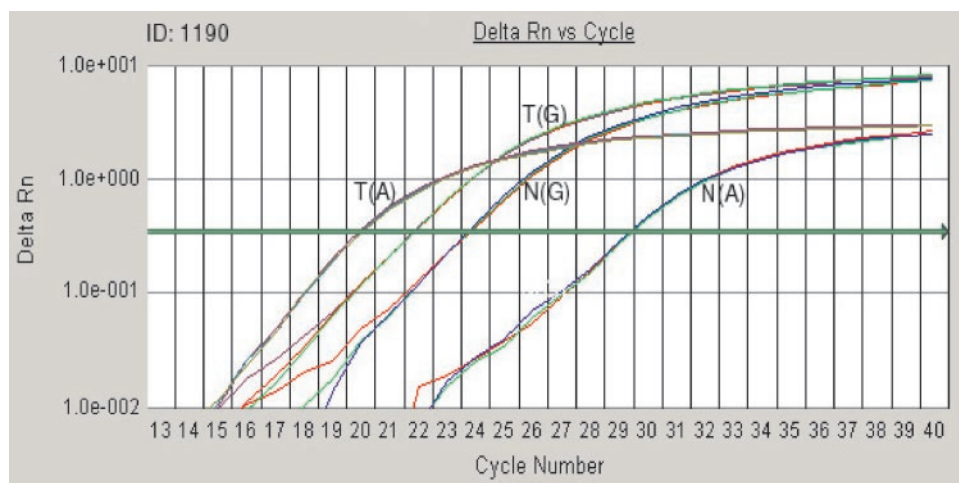


Fig. 2 Examples of amplification plot. Each sample was run in triplicate (orange, blue, and green lines). G = *GADPH*, A = *annexin I*, T = RNA from tumor tissues, and n = RNA from matched normal tissues. Case SHE1190 shows almost total loss of mRNA expression (tumor:normal expression ratio = 0.0006).

northern China. A58G was previously reported in a case-control study of type 2 diabetes in Finland and Sweden, where the frequency of the variant allele (G) was 0.05 in control subjects (14), much lower than we observed in healthy individuals (0.19), suggesting that the variant allele distribution of this SNP varies by ethnicity.

It seems likely that the reason we saw no significant relationship between genomic alterations on chromosome 9 and *annexin I* is because heterozygosity of these two SNPs was relatively low (29%), and homozygous deletions cannot be detected by SSCP, the technique used here. However, 81% of the 16 patients who were heterozygous showed intragenic allelic loss at one or both SNPs, including 10 (77%) who also had LOH at one or more of the microsatellite markers flanking *annexin I* that were tested. Thus, these results indicate that there is some level of genetic instability in esophageal squamous cell carcinoma patients, and additional study of these two SNPs in larger numbers of subjects is warranted to determine whether they are associated with increased susceptibility to esophageal squamous cell carcinoma in this high-risk population.

Quantitative RT-PCR identified decreased expression of *annexin I* mRNA in most esophageal squamous cell carcinoma. Thus far, only one other study of esophageal squamous cell carcinoma has reported decreased expression of *annexin I* RNA (in 17 of 24 esophageal squamous cell carcinomas), but expression levels were not quantitated in that study (23). To our

knowledge, the current study represents the first published data on *annexin I* mRNA using a quantitative real-time RT-PCR method in esophageal squamous cell carcinoma. We also noted that 19% of patients had increased expression of RNA of this gene. Possible explanations for this include: (a) our use of LCM DNA for mutation analysis without separation of RNA from well-differentiated and poorly differentiated foci; (b) direct extraction of RNA from frozen tumor tissue without LCM separation of well-differentiated from poorly differentiated and with potential admixture of normal tissue with tumor; and (c) general heterogeneity among patients.

Annexin I protein expression in esophageal squamous cell carcinoma showed a decrease in foci of poorly differentiated tumor cells but positive expression in the limited number of dysplastic and CIS lesions studied here. Future testing of protein expression with larger numbers of samples, as from biopsies with dysplasia or CIS, should help clarify these unexpected findings and aid in further understanding of annexin I protein expression in the development of esophageal squamous cell carcinoma.

The clinical relevance of the findings presented here stem from the advancement of our understanding of the mechanism of action of *annexin I*. These data strongly suggest that the loss of *annexin I* expression in squamous esophageal carcinogenesis is transcriptionally controlled and is unrelated to genomic mutations or epigenetic events. These data also provide confirmatory evidence regarding the high frequency of loss of expression

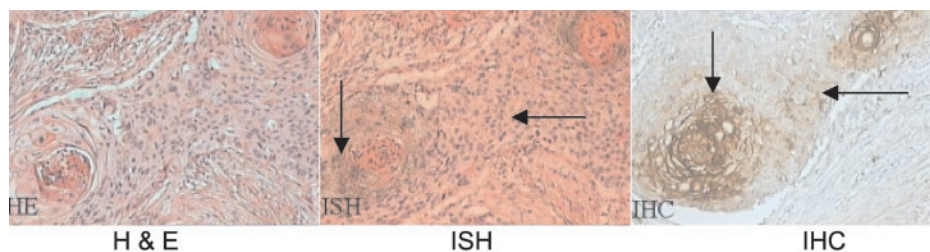


Fig. 3 *Annexin I* H&E (A), in situ hybridization (ISH; B), and immunohistochemistry (IHC; C) analysis of case SHE052: mRNA and protein expression varies with histologic differentiation such that increased expression is seen in well-differentiated foci (vertical arrows) and decreased expression is seen in poorly differentiated foci (horizontal arrows).

and establish *annexin I* as a solid candidate for additional testing as an early detection marker for esophageal squamous cell carcinoma.

There are several potential limitations in our approaches that merit comment. First, we did not have sufficient materials available to perform our DNA, RNA, and protein analyses on the same patients. Although there was substantial overlap in the patients evaluated for DNA and protein, we had no frozen tissue on the core 56 patients studied here, so we relied on a new set of patients for our mRNA analyses. Although the patients came from the same region of China and presented to the same cancer hospital, we cannot account for the degree of discordance that was introduced. Second, we may have underestimated the frequency of *annexin I* gene mutations because SSCP does not detect homozygous deletions. Third, we used LCM DNA for mutation analysis, but we did not separate DNA from well-differentiated and poorly differentiated areas and that may have masked differences between well- and poorly differentiated cellular foci for both DNA and RNA analyses.

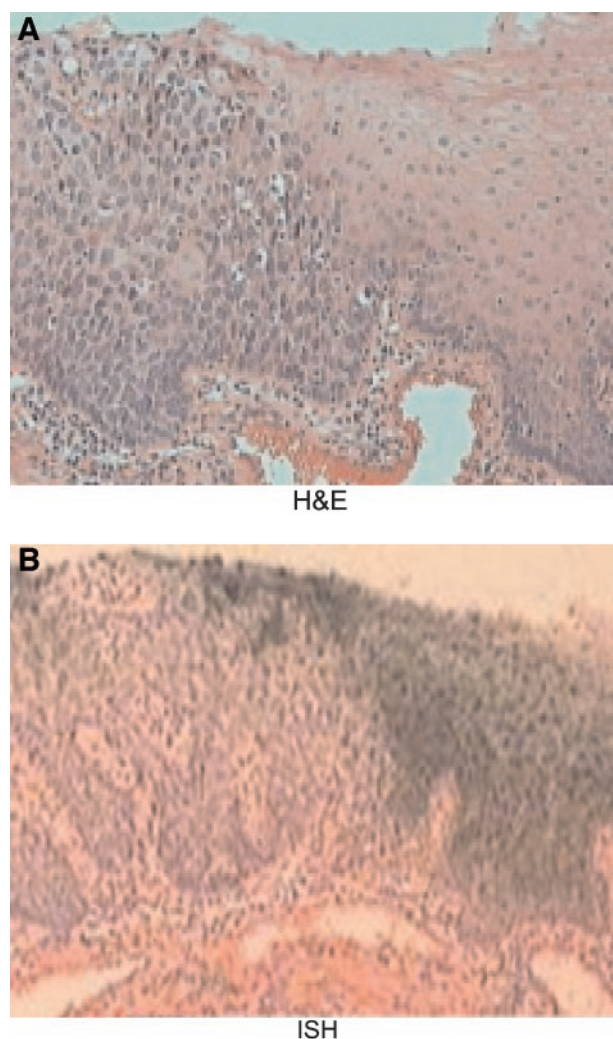


Fig. 4 *Annexin I* H&E (A) and ISH (B) analysis of case SHE488 showing a transition zone of normal esophageal epithelium to CIS with an associated change in expression.

Table 4 Comparison of chromosome 9 LOH and annexin I protein expression

Case	Sample origin	D9S1822	D9S1876
SHE 261	B	H	R
	N	H	R
	Annexin I (+) foci	H	L
	Annexin I (-) foci	H	L
SHE 57	B	R	R
	N	R	R
	Annexin I (+) foci	L	L
	Annexin I (-) foci	L	L
SHE 186	B	R	R
	N	R	R
	Annexin I (+) foci	L	L
	Annexin I (-) foci	L	L
SHE 52	B	R	R
	N	R	R
	Annexin I (+) foci	L	L
	Annexin I (-) foci	L	L
SHE 200	B	R	R
	N	R	R
	Annexin I (+) foci	L	L
	Annexin I (-) foci	L	L

Abbreviations: B, blood DNA; N, LCM DNA from adjacent normal tissue, annexin I (+/-) based on ISH result; R, retention; H, homozygous; L, loss of heterozygosity.

Fourth, we did not use laboratory techniques to identify changes in transcriptional or posttranslational modifications that may affect relevant downstream events and thus have no information on potential influences of these phenomena. Finally, the relatively small number of heterozygotes limited our ability to fully evaluate intragenic allelic loss.

One analysis we chose not to conduct, gene methylation, warrants mention given its frequent association with decreased gene expression in cancer. Gene methylation was deemed to be an unlikely silencing mechanism for *annexin I* because there are no CpG islands in either the promoter or coding region of this gene, the potential sites where gene methylation occurs. However, one recent study found that treating B-cell non-Hodgkin's lymphoma cell lines with 5-aza-2-deoxycytidine did result in reexpression of annexin I protein (24). It remains unclear whether this result reflects demethylation of genes upstream from *annexin I* or changes in *annexin I* itself.

In summary, intragenic allelic loss involving the *annexin I* gene occurs frequently, whereas somatic mutations are rare. However, loss of annexin I protein expression was confirmed, quantitated through mRNA expression analyses, and shown to be related to tumor cell differentiation. The most likely explanation for the loss of protein expression is an alteration in transcription. Thus, changes in *annexin I* mRNA and protein expression may be involved in the development of esophageal squamous cell carcinoma. The high prevalence of protein loss indicates that *annexin I* is a strong candidate for inclusion in a panel of early detection markers for esophageal squamous cell carcinoma.

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